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Phylogenetic Analysis of Marine Picoplankton Using rRNA Sequences.

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of the program is to analyze phylogenetically the dominant constituents of marine picoplankton in selected Atlantic and Pacific Oceans oligotrophic sites using 16S ribosomal RNA gene cloning and sequencing procedures. Additional goals are the development of rRNA-based hybridization probes for the phylogenetic analysis of single cells and the continued accumulation of 16S rRNA reference sequences. Recent efforts have resulted in the establishment of a recombinant DNA library of Sargasso Sea and northern Pacific picoplankton, the development of fluorescence-based, phylogenetic group-specific hybridization probes, and a continuing phylogenetic survey of cultivated cyanobacteria. Keywords: phylogeny, taxonomy,

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The purpose of the program is to analyze phylogenetically the dominant constituents of marine picoplankton in selected Atlantic and Pacific oligotrophic sites using 16S ribosomal RNA gene cloning and sequencing procedures. Additional goals are the development of rRNA-based hybridization probes for the phylogenetic analysis of single cells and the continued accumulation of 16S rRNA sequences. Recent efforts have resulted in the establishment of a recombinant DNA library of Sargasso Sea (Atlantic) and Northern Pacific picoplankton, the development of fluorescence-based, phylogenetic group-specific hybridization probes, and a continuing phylogenetic survey of cultivated cyanobacteria.

Progress Report Contract N00014-87-K-0813

Principal Investigator: Norman R. Pace

Title: Phlyogenetic Analysis of Marine Picoplankton Using Ribosomal RNA Sequencing

Project Goals:

The main goal of the program is the phylogenetic characterization of the dominant constituents among selected marine picoplankton populations using 16S ribosomal RNA (rRNA) gene cloning and sequencing. The analysis uses DNA extracted from the natural population, so it sidesteps the necessity of cultivating resident lifeforms. A second goal is the development of rRNA-based oligonucleotide hybridization probes suitable for the phylogenetic analysis of single cells, microscopically. A third goal is the continued accumulation of 16S rRNA sequences from cultivated organisms that are potentially related to picoplankton, in order to provide a reference data base of sequences for phylogenetic analyses.

Progress (Year 2):

A tangential flow filtration system employing 0.1μm pore concentration filters had been used to collect mixed picoplankton from an oligotrophic site in the Sargasso Sea. Mixed-organism DNA was isolated fragmented, cloned into phage λ (EMBL3) and screened for 16S rRNA genes. Although several rRNA clones were obtained, they were representative of only three organisms. A significant problem with the recombinant library was low yield of recombinants from the Sargasso sample. Subsequent experiments have traced this to an interference of ligation of DNA fragments to the phage λ arms (or other added DNAs). This problem is still under study, but it seems not to be a general problem: abundant rRNA clones were obtained in another tangential flow-concentrated sample from the north Pacific (ALOHA Station, a Global Ocean Flux Studies site north of Hawaii). These currently are under sequence analysis. Sequence analysis is proceeding more rapidly since the advent of polymerase chain reaction (PCR). The current protocol for screening rRNA gene-containing clones involves PCR amplification of rDNA in small recombinant phage lysates using oligonucleotide primers that are complementary to universally present, 16S rRNA sequences; then sequencing amplified product. Single dideoxynucleotide sequencing reactions are used to distinguish different clones, prior to further, standard ddNTP sequencing.

Good progress has been made toward devising hybridization probes for the phylogenetic analysis of single cells. These probes are synthetic oligodeoxynucleotides that are complementary to phylogenetic group-specific sequences in the 16S rRNA; hence there is a sufficient number of targets in each cell that probe binding to a single cell can be visualized by fluorescence microscopy. Oligonucleotides are synthesized to contain a 5'-amino group so that fluor isothiocyanates can be coupled to them. We have had good success using fluorescein- and rhodamine-labeled probes that readily distinguish eukaryotes, archaebacteria and eubacteria from one another in test mixtures; and several species-specific probes have been shown to distinguish their targets from close relatives. We are confident that these "phylogenetic stains" will prove broadly applicable, however, there currently is difficulty in achieving sufficient probe-binding to organisms in natural populations, probably because of low ribosome contents (lower growth rates than laboratory cultures). Efforts are underway to improve the fluorescence yield of probes, an important goal for the study of naturally-occurring microbial populations. The probes have recently been used to identify uncultivatable organisms, to trace an insect symbiont in natural populations, and to study the tissue distribution of a bacterial symbiont in shipworms.

A fairly comprehensive, 16S rRNA-based phylogenetic survey of diverse cyanobacteria (29 new sequences) was recently completed in the effort to expand the data base of reference sequences for the picoplankton analysis. Significant new findings are that the cyanobacteria are a relatively young group of eubacteria, that many diverse forms of cyanobacteria diverged within a short span of evolutionary time, and that the green chloroplasts are a subgroup within the cyanobacteria, not a sister lineage as previously was thought. The phylogenetic analysis points out substantial problems with the traditional, morphology-based taxonomy of cyanobacteria. 16S rRNA sequencing also resolved the relationship of the "prochlorophyte" *Prochlorothrix hollandica* to green chloroplasts. They are not specific relatives.

Work Plan (Year 3):

Analysis of the DNA and rRNA clones derived from the picoplankton collections will continue. Major effort, pending further collection from the Atlantic, will focus on the Pacific sample. Oligonucleotide hybridization probes based on the sequences will be synthesized in order to identify the morphotypes in the population that correspond to the rRNA sequences.

Further effort will be invested toward increasing the fluorescence yield of single cell, phylogenetic group-specific hybridization probes. Methods will focus on addition of multiple fluorescent groups to the oligonucleotide probes, using chemical couplings (e.g. by attaching multiple aliphatic amino groups to the oligonucleotides) as well as "sandwich" methods (e.g. using biotinylated probes and staining with avidin-fluor conjugates).

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